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Research Note

Species-Specific Identification of *Penicillium* Linked to Patulin Contamination†

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ABSTRACT

Certain species of *Penicillium* have been reported to produce the mycotoxin patulin, and research was undertaken to identify these with the use of oligonucleotide primer pairs. Species examined were found in food, plants, and soil and were reported to produce patulin. *Penicillium expansum* is the most commonly detected species linked to the presence of patulin in apple juice. At least 10 different enzymes are involved in the patulin biosynthetic pathway, including the isoepoxydon dehydrogenase (*idh*) gene. Based on nucleotide sequences previously determined for the *idh* gene in *Penicillium* species, PCR primers were designed for the species-specific detection of patulin-producing species. The 5′ primers were based on differences in the second intron of the *idh* gene. To ensure that the primer pairs produced a PCR product restricted to the species for which it was designed, and not to unrelated species, all of the primer pairs were tested against all of the *Penicillium* species. With one exception, it was possible to detect a reaction only with the organism of interest. The primer pair for *Penicillium griseofulvum* also amplified DNA from *Penicillium dipodomyicola,* a closely related species; however, it was possible to distinguish between these two species by doing a second amplification, with a different primer pair specific only for *P. dipodomyicola.* Consequently, with different primer sets, it was possible to identify individual patulin-producing species of *Penicillium.*

Patulin, a toxic secondary metabolite, is produced by certain species of *Aspergillus, Byssochlamys,* and *Penicillium. Penicillium expansum* is the fungus most commonly associated with patulin contamination of apple juice and apple products. Patulin has been reported to have mutagenic, carcinogenic, and teratogenic properties, but this is still a matter of debate *(6).* For example, only a low level of benign tumors was produced as a result of long-term testing of patulin directly applied to the forestomach of Sprague-Dawley rats *(1).* Fourteen different species in the genus *Penicillium* have been reported to produce patulin *(5),* although only six species (*P. expansum, P. griseofulvum, P. dipodomyicola, P. carneum, P. paneum,* and *P. sclerotigenum*) have been found in food. Species associated with food products are of interest because they represent a food safety concern. *Penicillium* species associated with plants and soil that produce patulin include *P. clavigerum, P. concentricum, P. coprobium, P. gladioli, P. glandicola,* and *P. vulpinum.*

In this study, we have developed a PCR-based method for identification of patulin-producing species, which is specific to the isoepoxydon dehydrogenase (*idh*) gene sequence. IDH *(4),* the seventh of 10 enzymes in the direct pathway involved in the synthesis of patulin *(12),* was the focus of our study. Biochemical studies elucidating the pathway were previously reported *(4, 7, 9–11).* Sequences of only two genes (*6-msas* and *idh*) of the patulin pathway enzymes were the first to be deposited in GenBank, although recent deposits of additional genes have been made *(12).* The first enzyme in the patulin biosynthetic pathway is 6-methylsalicylic acid synthase *(2),* which forms 6-methylsalicylic acid by condensation of one molecule of malonyl-CoA and three molecules of acetyl-CoA. IDH *(4)* in the terminal portion of the patulin pathway is responsible for the conversion of isoepoxydon to phyllostine.

There have been many attempts to develop rapid methods for the detection of patulin. There has been no success to date for generating antibodies capable of specifically detecting patulin. The methods for quantitation of patulin require cleanup protocols and then the use of expensive high-performance liquid chromatography equipment by trained analysts. Processors of apple juice would be assisted by having a means for detecting the presence of patulin-producing fungi at an early stage in production. The majority of the apple juice sold in the United States is imported and shipped as apple juice concentrate in large 55-gal (208-liter) containers. For apple juice concentrate to be imported into the United States, it must be verified that, when diluted to regular strength, it contains less than 50 ng of patulin per

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† Names of equipment and chemical supplies are necessary to report factually on experimental methods; however, the U.S. Department of Agriculture neither guarantees nor warrants the standard of the products, and the use of the name by U.S. Department of Agriculture implies no approval of the products to the exclusion of others that may also be suitable.

TABLE 1. Penicillium *species for which primers were designed for specific amplification of the* idh *gene*

Strain designation	*Penicillium* species	Common sources of species
NRRL 2159A	*P. griseofulvum*	Cereals, pasta
NRRL 3523	*P. griseofulvum*	Cereals, pasta
NRRL 5256	*P. griseofulvum*	Cereals, pasta
NRRL 35258	*P. griseofulvum*	Cereals, pasta
NRRL 2304	*P. expansum*	Apple juice, apples
NRRL 6069	*P. expansum*	Apple juice, apples
NRRL 32289	*P. expansum*	Apple juice, apples
NRRL 32293	*P. expansum*	Apple juice, apples
NRRL 35231	*P. expansum*	Apple juice, apples
NRRL 35259	*P. expansum*	Apple juice, apples
NRRL 1003	*P. clavigerum*	Soil, not in foods
NRRL 1004	*P. clavigerum*	Soil, not in foods
NRRL 25159	*P. paneum*	Rye bread
NRRL 25162	*P. paneum*	Rye bread
NRRL 3461	*P. sclerotigenum*	Yams
NRRL 22813	*P. sclerotigenum*	Yams
NRRL 13626	*P. coprobium*	Rarely in foods
NRRL 1002	*P. vulpinum*	Soil, insects, dung
NRRL 2031	*P. vulpinum*	Soil, insects, dung
NRRL 2034	*P. concentricum*	Rarely in foods
NRRL 35582	*P. dipodomyicola*	Rice, soil
NRRL 35583	*P. dipodomyicola*	Rice, soil
NRRL 25168	*P. carneum*	Rye bread, beer, wine
NRRL 25170	*P. carneum*	Rye bread, beer, wine
NRRL 985	*P. glandicola*	Soil, silage
NRRL 2036	*P. glandicola*	Soil, silage
NRRL 938	*P. gladioli*	*Gladiolus* bulbs
NRRL 939	*P. gladioli*	*Gladiolus* bulbs

liter of apple juice, the regulatory level set by the U.S. Food and Drug Administration. Apple juice concentrate must be diluted to regular strength before it can be analyzed.

Use of a rapid species-specific DNA detection method will enable the identification of *Penicillium* in apple juice concentrate before its use for the production of single-strength apple juice. This would alert juice manufacturers to the possible presence of patulin, as well as provide an accurate means for identification of any contaminating penicillia. We examined 28 strains from 12 different species of *Penicillium* known to produce patulin to design species-specific primers for PCR identification of these fungi. Research had previously been undertaken in our laboratory to determine the nucleotide sequence of the *idh* gene of the patulin biosynthetic pathway to compare the different strains of the *Penicillium* species known to have the ability to synthesize patulin *(3).* The presence of a particular patulin biosynthetic gene does not indicate with certainty that patulin will be present, but the detection of fungi capable of synthesizing patulin would alert apple juice producers of a potential problem.

MATERIALS AND METHODS

Fungal strains and growth conditions. The 28 strains of 12 different *Penicillium* species used in this study are listed in Table 1, along with their common habitats. The *Penicillium* cultures were grown on potato dextrose agar plates for approximately 2 weeks at 25°C prior to isolation of total genomic DNA.

DNA extraction. Total genomic DNA from the 28 strains of 12 different *Penicillium* species was isolated in the following manner. Approximately 2 to 3 ml of 70% ethanol was added to each plate, and the surface growth was brought into suspension with a sterile rod bent at a 90° angle. The suspension was placed into a 1.5-ml tube and centrifuged for 5 min to precipitate the cellular material; the supernatant was discarded. Three hundred seventy-five microliters of DNA extraction buffer (200 mM Tris, 250 mM NaCl, 25 mM EDTA [pH 8.5], and 0.5% sodium dodecyl sulfate) and 125 μl of glass beads were added to the pellet, and the tubes were vortexed for 5 to 10 min with a TurboMix (Scientific Industries, Bohemia, N.Y.). Three hundred fifty microliters of 2× CTAB (cetrimonium bromide) buffer was added to the tube of broken cells, and the mixture was vortexed for approximately 30 s. Three hundred fifty microliters of chloroform was added and carefully vortexed to emulsify the solution containing the broken cells, which was then centrifuged for 10 min at maximum speed to separate the emulsion. The upper aqueous phase was carefully removed and placed into a new 1.5-ml tube. Five hundred microliters of −20°C isopropanol was added to precipitate the DNA. After centrifugation for 5 min, the supernatant was discarded. The DNA pellet was washed with 1,000 μl of 70% ethanol and centrifuged for 3 min, and the supernatant was discarded. The DNA pellet was then dissolved in 100 μl of TE buffer (10 mM Tris and 1 mM EDTA [pH 8.0]). A 1:10 dilution of stock DNA in TE/10 buffer was used as the working stock of DNA.

Design of species-specific primers. With the *idh* sequences of the *Penicillium* strains previously determined *(3),* species-specific primers for each of the 12 species were designed. Intron 1 (Fig. 1) did not have much diversity; hence, the 5′ primers were designed on the basis of intron 2 for all 12 of the *Penicillium* species (Fig. 2). To achieve specificity for the 5′ primer for *P. griseofulvum,* an additional five nucleotides (beyond the sequence of intron 2) were added at the 3′ end. To achieve specificity for the 5′ primer for *P. glandicola,* it was necessary to add five nucleotides (not part of intron 2, but immediately upstream of intron 2) at the 5′ end of the primer. The 3′ primers were designed from an area within the last 80 bp of the *idh* gene. This was done so that the expected sizes of the PCR products would be similar (467 to 502 bp) (Table 2).

Amplification of the *idh* gene. Concentrations of DNA and primers were determined and verified by agarose gel electrophoresis before doing PCR. Symmetric amplification was performed in a 96-well plate. The PCR reaction mixture per well contained the following: 6.3 μl of dH_2O, 4 μl of 10× PCR buffer, 7.2 μl of deoxynucleotide mix (1.25 mM), 1.0 μl of external 5′ end primer (10 pmol/μl), 1.0 μl of external 3′ end primer (10 pmol/μl), 0.4 μl of *Taq* polymerase (Sigma-Aldrich, St. Louis, Mo.), and 20 μl of genomic DNA (approximately 10 ng/μl) in TE/10. The plate was covered with a rubber mat, vortexed, and then spun briefly in a centrifuge to collect the liquid in the bottom of the wells. The plate was put into a PCT-100 Programmable Thermal Controller (MJ Research, Inc., Waltham, Mass.) and run with the following program: 94°C for 1 min, 58°C for 55 s, and 72°C for 2 min (35 times); 94°C for 1 min; 58°C for 55 s; and 72°C for 10 min (final extension step). Each strain was amplified at least twice with each set of primers. After amplification, all PCR products were visualized with agarose gel electrophoresis. To determine optimal conditions for amplification, it was necessary to test

FIGURE 1. *Alignment of sequences of intron 1 in the* idh *gene of the* Penicillium *species. The number preceding each strain is the accession number in the ARS Culture Collection (NRRL).*

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2159A P.griseofulvum    GTAAGGAGAAT-AA-G-T-AAAA-TAT----ATCA----AGCTGCAT--CCGACTAATATGGGAATCAG
 3523 P.griseofulvum    GTAAGAAGATT-AA-G-T-AAAA-TAT----ATCA----AGCTGCAT--CTCACTAACATGGGAATTAG
 5256 P.griseofulvum    GTGAGAAGAAT-AA-G-T-AAGC-TAT----ATCA----AGCTGCAT--CCGACTAATATGGGAATCAG
35258 P.griseofulvum    GTGAGAAGAAT-AA-G-T-AAGC-TAT----ATCA----AGCTGCAT--CCGACTAATATGGGAATCAG
35582 P.dipodomyicola   GTAAGAAGAAT-AA-G-T-AAAA-TTT----ATCA----AGCTGCAT--CCGACTAACATGGGAATTAG
35583 P.dipodomyicola   GTAAGAAGAAT-AA-G-T-AAAA-TTT----ATCA----AGCTGCAT--CCGACTAACATGGGAATTAG
35231 P.expansum        GTAAGAAGAATCAA-GATCAAGA-AAAAAACAT-ACC-TAGTTGTGT--CCGACTAACGCGGGAACTAG
 6069 P.expansum        GTAAGAAGAATCAA-GATCAAGA-AAAAAACAT-ACC-TAGTTGTGT--CCGACTAACGCGGGAACTAG
 2304 P.expansum        GTAAGAAGAATCAA-GATCAAGA-AAAAAACAT-ACC-TAGTTGTGT--CCGACTAACGCGGGAACTAG
32289 P.expansum        GTAAGAAGAATCAA-GATCAAGA-AAAAAACAT-ACC-TAGTTGTGT--CCGACTAACGCGGGAACTAG
32293 P.expansum        GTAAGAAGAATCAA-GATCAAGA-AAAAAACAT-ACC-TAGTTGTGT--CCGACTAACGCGGGAACTAG
35259 P.expansum        GTAAGAAGAATCAA-GATCAAGA-AAAAAACAT-ACC-TAGTTGTGT--CCGACTAACGCGGGAACTAG
 1003 P.clavigerum      GTAGGAA-AATGAC-AAAATTATGAAAAA-GAT-------GT-GTG--CCCAGCTAACACAGAAACTAG
 1004 P.clavigerum      GTAGGAAAAATGACAAAAATTATGAAAAA-GAT-------GT-GTG--CCCAGCTAACTCAGAAACTAG
25159 P.paneum          GTAAGCGAAAA-A-AGAAA--GAAAAGAAAAATATATCAACCTATGT-CCGA-CTAACGCTGGA-CTAG
25162 P.paneum          GTAAGCGAAAA-A-AGAAA--GAAAAGAAAAATATATCAACCTATGT-CCGA-CTAACGCTGGA-CTAG
 3461 P.sclerotigenum   GTAAGAAAAATGACAAAAA--AACAAGAAAAGT-T-CATCAAGTTGTGTTCCACTAACGAGGGAACTAG
22813 P.sclerotigenum   GTAAGAAAAATGACAAAAA--AACAAGAAAAGT-T-CATCAAGTTGTGTTCCACTAACGAGGGAACTAG
 1002 P.vulpinum        GTAAGAAAAAT----AAAA-------GAAAAAAGTACATCAATTTGCATCCGACTAACGCGGGGTCTAG
 2031 P.vulpinum        GTAAGAAAAAT----AAAA-------GAAAAAAGTACATCAATTTGCATCCGACTAACGCGGGGTCTAG
 2034 P.concentricum    GTAAGAAAA-C---AA----------GTAAAATC--CATCATGCTGCGTCTGACTGACGCGGGTACTAG
25168 P.carneum         GTAAGTGAGAGG---GAAA-----TAT----ATCA------ACTTAGGTCTGACTAACGCGGG-ACTAG
25170 P.carneum         GTAAGTGAGAGG---GAAA-----TAT----ATCA------ACTTAGGTCTGACTAACGCGGG-ACTAG
  985 P.glandicola      GTCAGGAAA-TGA--G---AAAA-TA----CATCA------AGCTGGGTCCGACTGACGTGGAGGTTAG
 2036 P.glandicola      GTAAGAAAA-TGA--G-T-AAAA-TA----CATCA------AGCTGGGTCCGACTGACGTGGAGATTAG
  938 P.gladioli        GTAAGAAAAAATAA--G---AAAA-------CA-G-G--TCAAGCTACGTCCAACTAACCCGGGAACTAG
  939 P.gladioli        GTAAGAAAAAATAA--G---AAAA-------CA-G-G--TCAAGCTACGTCCAACTAACCCGGGAACTAG
13626 P.coprobium       NNAAGGGAAAAAAAAAAAAAGAAAAAGAGAAGACTACATCAACCTGCACCCGACTAACACAGATACTAG
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several different primer sets and different amplification temperatures so that only one band appeared on the gel.

Characterization of *idh* gene amplicons. The presence of a DNA fragment of 467 to 502 bp was taken as a positive result for the species-specific amplicons. The following are the lengths of species-specific amplicons: *P. glandicola* (467 bp), *P. griseofulvum* (472 bp), *P. coprobium* (477 bp), *P. expansum* (480 bp), *P. paneum* (482 bp), *P. carneum* (486 bp), *P. concentricum* (489 bp), *P. clavigerum* (492 bp), *P. vulpinum* (494 bp), *P. gladioli* (495 bp), *P. sclerotigenum* (498 bp), and *P. dipodomyicola* (502 bp).

RESULTS

The goal of the research was to develop a means to identify specific species of *Penicillium.* Except for a single case, there was no cross-reactivity with other *Penicillium* species. Consequently, species can be identified by setting up a standard PCR reaction with the primers specified. Each set of primers was tested with each of the strains of *Penicillium* species to determine the conditions for specific recognition. The sequence of the *idh* gene had previously been determined for the 28 strains representing the 12 different *Penicillium* species reported to produce the mycotoxin patulin *(3).* The amino acid sequences of the 12 species were aligned and compared after the two introns had been removed and the DNA sequences translated. There were many nucleotide differences present in the *idh* gene sequence among these species of *Penicillium,* but the vast majority of the differences did not affect the amino acid sequences because they were present in the third position of the codon.

Because of numerous nucleotide differences in the *idh* genes, these sequences were utilized to identify species-specific primers that could be used for rapid, accurate identification of patulin-producing species of *Penicillium.* This was possible in all but one case. In this single case, the primer set for *P. griseofulvum,* because it was closely related to *P. dipodomyicola (3),* amplified the strains of both *P. griseofulvum* and *P. dipodomyicola*; the primer set, however, specific for *P. dipodomyicola,* amplified only the two strains of *P. dipodomyicola* and not strains of *P. griseofulvum.* Thus, it was possible to run two separate amplifications of genomic DNA to distinguish between strains of *P. griseofulvum* and *P. dipodomyicola.* First, the *P. griseofulvum*–specific primer set was used to identify strains of both *P. griseofulvum* and *P. dipodomyicola*; the size of the bands for both *P. griseofulvum* and *P. dipodomyicola* was 472 bp when the *P. griseofulvum*–specific primer set was used. Second, the *P. dipodomyicola*–specific primer set was used to identify only the *P. dipodomyicola* strains; the size of the band was 502 bp. The important distinction is the pres-

FIGURE 2. *Alignment of sequences of intron 2 in the* idh *gene of the* Penicillium *species. The number preceding each strain is the accession number in the ARS Culture Collection (NRRL).*

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2159A P.griseofulvum    GTAAGTTATTTTCCCAGAATCCACTGG-TTTAAAAGCATACTGAC-AAGCTATAG
 3523 P.griseofulvum    GTAAGTTATTTCCCCAAAATTCACTGG-TTTAAAATCATACTAAC-AAGCTATAG
 5256 P.griseofulvum    GTAAGTTATTTTCCCAGAATCCACTGG-TTTAAAAGCATACTGAC-AAGCTATAG
35258 P.griseofulvum    GTAAGTTATTTTCCCAGAATCCACTGG-TTTAAAAGCATACTGAC-AAGCTATAG
35582 P.dipodomyicola   GTAAGTGATTTCCCTGGCATAAATTGGTTTAAAAAACATACTGAC-AAGCCGTAG
35583 P.dipodomyicola   GTAAGTGATTTCCCTGGCATAAATTGGTTTAAAAAACATACTGAC-AAGCCGTAG
35231 P.expansum        GTGATTGATTTTCTTAGAATTTCTTAT-TTAAAATGTGTACTGAC-TGGTCGCAG
 6069 P.expansum        GTGATTGATTTTCTTAGAATTTCTTAT-TTAAAATGTGTACTGAC-TGGTCGCAG
 2304 P.expansum        GTGATTGATTTTCTTAGAATTTCTTAT-TTAAAATGTGTACTGAC-TGGTCGCAG
32289 P.expansum        GTGATTGATTTTCTTAGAATTTCTTAT-TTAAAATGTGTACTGAC-TGGTCGCAG
32293 P.expansum        GTGATTGATTTTCTTAGAATTTCTTAT-TTAAAATGTGTACTGAC-TGGTCGCAG
35259 P.expansum        GTGATTGATTTTCTTAGAATTTCTTAT-TTAAAATGTGTACTGAC-TGGTCGCAG
 1003 P.clavigerum      GTGATTGATTCCCCTGACACTGCCTGG-TTCACATGAATACTAAC-TGCCCACAG
 1004 P.clavigerum      GTGATTGATTCCCCTGACACTTCCTGG-ATCACATGAATATTAAC-TGCCTACAG
25159 P.paneum          GTGATTGATTTCCCCAGCAGTTCTTGG-TTAGAATACACACTGAC-TGGCCGTAG
25162 P.paneum          GTGATTGATTTCCCCAGCAGTTCTTGG-TTAGAATACACACTGAC-TGGCCGTAG
 3461 P.sclerotigenum   GTGATTGCTTTTCCTGAAATTGCCTGG-TTAACATTCATACTAAC-TGCCCGCAG
22813 P.sclerotigenum   GTGATTGCTTTTCCTGAAATTGCCTGG-TTAACATTCATACTGAC-TGCCCGCAG
 1002 P.vulpinum        GTGATTGATTTTCCCTACATCTTCTGG-TTCAAATTCATACTGAC-TGGCCGCAG
 2031 P.vulpinum        GTGATTGATTTTCCCTACATCTTCTGG-TTCAAATTCATACTGAC-TGGCCGCAG
 2034 P.concentricum    GTGATTGATCCCCCGGGAGTCCTCTGG-TTAAAATTAATACTGAC-TGGCCGCAG
25168 P.carneum         GTGATTGATTT-CCCGGCACTCCCTAG-TTAGAATGCAAACTGAC-TGGCTGTAG
25170 P.carneum         GTGATTGATTT-CCCGGCACTCCCTAG-TTAGAATGCAAACTGAC-TGGCTGTAG
  985 P.glandicola      GT-A---ATTTCCCCAGAATCCTATAG-TTGAAATGCAGACTGACATGGCCGCAG
 2036 P.glandicola      GT-A---ATTTCCCCAGAATGCTATA- TTTATATACAAAATGACATGGCCGCAG
  938 P.gladioli        GTGATTGGTTTCCCTGACATCTCCTGG-TTCAAATGCATACTAAC-TGGCCGCAG
  939 P.gladioli        GTGATTGGTTTCCCTGACATCTCCTGG-TTCAAATGCATACTAAC-TGGCCGCAG
13626 P.coprobium       GTAATTGATCTTCCCGGAATCCTCTGG-TTAGAATTCACACTAAC-TAGCCGCAG
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TABLE 2. *Oligonucleotide primers used in this study for species-specific amplification of the* idh *gene*

Penicillium species	5′ primer sequence	3′ primer sequence	Annealing temp (°C)	Expected product size (bp)
P. griseofulvum	5′CATACTRACAAGCTATAGCATCA	5′CAACATATTCGTGCCAGAAACG	58	472
P. expansum	5′AATGTGTACTGACTGGTCGCAG	5′CAACCAACATATTCGTGCCTGAC	58	480
P. clavigerum	5′GATTCCCCTGACACTKCCTG	5′AGTGCCCGTMACGAAGCTCGACAG	58	492
P. paneum	5′GAATACACACTGACTGGC	5′TCAACCAACACATTCGTACCAGAC	58	482
P. sclerotigenum	5′GCCTGGTTAACATTCATAC	5′TCCGCCATCGACCAGCACGTTCGTG	58	498
P. coprobium	5′GAATTCACACTAACTAGCCG	5′CAACATATTCGTGCCCGAGAC	58	477
P. vulpinum	5′CTACATCTTCTGGTTCAAATTC	5′CCAACAAATTTGTGCCGGACAC	58	494
P. concentricum	5′GGAGTCCTCTGGTTAAAATTAATACTGACTG	5′CATATTCGTCCCCGAGACAAAGCTG	58	489
P. dipodomyicola	5′GATTTCCCTGGCATAAATTGG	5′CAACATATTCGTGCCAGAAACG	58	502
P. carneum	5′CTAGTTAGAATGCAAACTGAC	5′ACCAACATATTCGTGCCAGATAC	58	486
P. gladioli	5′TGACATCTCCTGGTTCAAATGCATAC	5′ACCAACATATTCGTGCCCGTCAC	58	495
P. glandicola	5′CAATGGTAATTTCCCCAGAAT	5′TGCCCGACACGAAGCTGGCCAG	58	467

ence or absence of a reaction occurring with the specific set of primers and the presence of a band of an appropriate size, not just on the size of the band on the gels. The result for all reactions with genomic DNA and the corresponding species-specific primer set was that the only bands present were those of the specific species for which they had been designed to amplify, and the bands were of the expected size. If apple juice processors or importers were only concerned with the potential presence of *P. expansum,* it would only be necessary to run one reaction with the primers specific for *P. expansum,* along with a positive control in a separate reaction in which DNA from a *P. expansum* control strain is analyzed. Alternatively, if they were concerned about the possible presence of strains of *Penicillium* that have been found in food, it would be necessary to do a total of six reactions for each sample, in addition to the corresponding positive controls. Consequently, by means of the primer pairs listed in Table 2, it was possible to identify accurately the 12 patulin-producing species examined in this study.

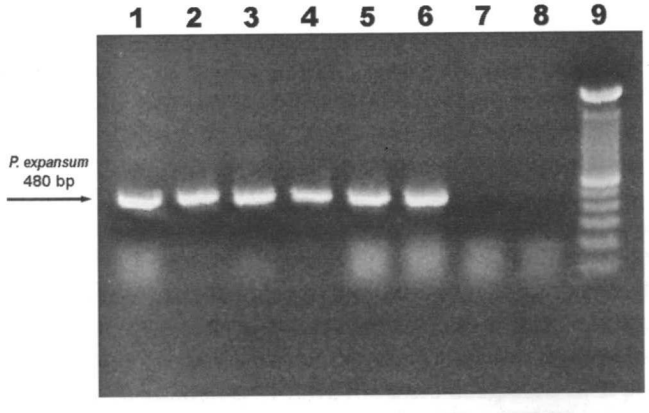

FIGURE 3. *Agarose gel electrophoresis of PCR-amplified products. Lane 1,* P. expansum *(NRRL 32293); lane 2,* P. expansum *(NRRL 35231); lane 3,* P. expansum *(NRRL 6069); lane 4,* P. expansum *(NRRL 2304); lane 5,* P. expansum *(NRRL 32289); lane 6,* P. expansum *(NRRL 35259); lane 7,* P. glandicola *(NRRL 985); lane 8,* P. coprobium *(NRRL 13636); lane 9, 100-bp ladder DNA marker. Samples from which the* idh *gene from* Penicillium *species were amplified.*

Results of a representative agarose gel electrophoresis of PCR amplicons for strains of *P. expansum* are shown in Figure 3. Extensive efforts were made to test each primer set with all of the strains (data not shown) to ensure that the primer pairs were species-specific. Because *P. expansum* is the major species responsible for producing patulin and has been identified in apples, apple juice, and products containing apples, it was important to show that all of the *P. expansum* strains were identified by the primer pair specific for *P. expansum* and neither of the two unrelated strains (*P. glandicola* and *P. coprobium*) (Fig. 3).

DISCUSSION

Design of species-specific primers to identify the presence of species of *Penicillium* was undertaken on the basis of the *idh* sequences for identification of the presence of *Penicillium* species having the ability to synthesize the mycotoxin patulin. The *idh* sequence codes for the enzyme IDH, the seventh gene in the patulin biosynthetic pathway. Each of the 5′ primers was designed on the basis of the sequence present in intron 2, where maximal nucleotide diversity was observed. An area within the last 80 bp of the *idh* gene was selected for the design of the 3′ primers.

The specific purpose of our study was to identify the presence of fungi known to produce patulin with species-specific primers for the *idh* gene. This goal was realized through the development of primer pairs for all 12 species studied. Recently, *P. formosanum* and *P. marinum,* two new patulin-producing species of *Penicillium,* were described *(8).* It is not likely that they would be detected in food products because they are found in dung and coastal sand, respectively. When cultures become available, primers will be developed for the detection of these species as well.

It would certainly be a good idea to include a separate reaction with DNA from a *P. expansum* control strain in each PCR assay when a sample is being checked. Having a positive control would strengthen the assay. These primers can be used to confirm the presence of penicillia in food systems and mixed cultures. Consequently, the detection system developed in the present study would be useful both

for processors of apple products and for studies involving risk analysis.

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